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Naringin abrogates osteoclastogenesis and bone resorption via the inhibition of RANKL-induced NF-κB and ERK activation

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ABSTRACT

Osteolytic bone diseases including osteoporosis are commonly accompanied with enhanced osteoclast formation and bone resorption. Naringin, a natural occurring flavonoid has been found to protect against retinoic acid-induced osteoporosis and improve bone quality in rats. Here, we showed that naringin perturbs osteoclast formation and bone resorption by inhibiting RANK-mediated NF-κB and ERK signaling. Naringin suppressed gene expression of key osteoclast marker genes. Naringin was found to inhibit RANKL-induced activation of NF-κB by suppressing RANKL-mediated IκB-α degradation. In addition, naringin inhibited RANKL-induced phosphorylation of ERK. This study identifies naringin as an inhibitor for osteoclast formation and bone resorption, and provides evidence that natural compounds such as naringin might be beneficial as an alternative medicine for the prevention and treatment of osteolysis.

1. Introduction

Enhanced osteoclast formation and bone resorption is an underlining mechanism of osteolytic bone diseases including osteoporosis, Paget's disease of bone, bone metastatic diseases, erosive arthritis, aseptic bone loosening and non-union. Understanding the molecular processes by which osteoclasts are formed and activated, and identifying the pharmacological intervention to perturb these processes might contribute to the prevention and treatment of osteoporosis.

Osteoclasts are the principal cells responsible for bone resorption and the main targets of current anti-resorptive drugs. The receptor activator of nuclear factor-kappa B (NF-κB) (RANK) ligand (RANKL) is a key cytokine obligatory for osteoclast formation, activation and survival [1,2]. The interaction of RANKL with RANK results in a cascade of intracellular events including NF-κB, AKT, MAPKs, NFAT, ionic calcium and calcium/calmodulin-dependent kinase. Among these pathways, NF-κB signaling has been shown to play an essential role in osteoclastogenesis [3,4].

Naringin, a natural occurring flavonoid is the major active component found in grapefruit and other related citrus. It inhibits dihydropyridine oxidation and aflatoxin B1 activation in human liver microsomes, and might have cancer chemoprevention properties [5]. It also protects against radiation-induced chromosomal damage in mouse bone marrow cells [6,7], and lomefloxacin-induced genomic instability [8]. Interestingly, naringin has been shown to protect against retinoic acid-induced osteoporosis in Sprague Dawley rats [9] and improve bone quality in orchidectomized male rats [10]. However, the cellular and molecular mechanism by which naringin offers protective effects against bone loss is not clear.

In search of natural compounds that exhibit pharmacologically inhibitory effects on osteoclasts, we have found that naringin inhibits osteoclast formation and bone resorption. Furthermore, we demonstrated that naringin inhibits RANKL-induced NF-κB activation, IκB-α degradation and ERK phosphorylation, by and large correlating its inhibitory effect on osteoclastogenesis and bone resorption.

Abbreviations: BMM, bone marrow macrophages; ERK, extracellular receptor kinase; GCT, giant cell tumour; M-CSF, macrophage-colony stimulatory factor; NF-κB, nuclear factor kappa B; OCL, osteoclast like cells; RANKL, receptor activator of nuclear factor kappa B ligand; RT-PCR, reverse transcriptase-polymerase chain reaction

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2. Materials and methods

2.1. Media and reagents

Alpha modified of Eagles Medium (α-MEM) and fetal bovine serum (FBS) was purchased from TRACE (Sydney, Australia). Naringin (>95% purity) was purchased commercially from Sigma–Aldrich (Sydney, Australia) and dissolved in Dimethyl sulfoxide (DMSO). Luciferase assay system was obtained from Promega (Sydney, Australia). Recombinant GST-rRANKL protein was expressed and purified as previously described [2].

2.2. Cell culture

RAW264.7 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). RAW264.7 cells were grown as per American Type Culture Collection guidelines in α-MEM supplemented with 10% heat inactivated FBS, 2 mM L-glutamine and 100 U/ml penicillin/streptomycin (complete α-MEM). Primary bone marrow macrophages were grown in α-MEM supplemented with 10% heat inactivated FBS, 2 mM L-glutamine and 100 U/ml penicillin/streptomycin with the addition of macrophage-colony stimulating factor (M-CSF, 25 ng/ml). All cell cultures were maintained in 5% CO_2 at 37 °C.

2.3. In vitro osteoclastogenesis assay

Osteoclast-like cells were generated using an established pro-osteoclastic system as previously described [11]. In brief, freshly isolated bone marrow macrophages (BMM) from C57/BL6 mice were cultured with M-CSF (25 ng/ml) for the first 3 days. BMM were then seeded onto a 96 well plate (8×10^3 cells/well) with complete α-MEM containing M-CSF (25 ng/ml) and GST-rRANKL (100 ng/ml). Medium and naringin was replaced every 2–3 days and after 7 days, cultures were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 10 min at room temperature and then washed four times with PBS. Fixed cells were stained for tartrate-resistant acid phosphatase (TRACP) using the Diagnostic Acid Phosphatase kit (Sigma) according to the manufacturer's instructions and TRACP positive multinucleated cells (>3 nuclei) were scored as osteoclast-like (OCLs) cells.

2.4. Immunofluorescent staining and confocal microscope

For immunofluorescence studies, 1×10^4 RAW264.7 cells were plated onto a 6-well plate pre-seeded with five coverslips. After overnight of incubation, cells were stimulated with RANKL (100 ng/ml) for 5 days to induce osteoclast formation in the absence or presence of naringin (0.1 and 0.5 mM). Cells were then washed twice with 1 × PBS, fixed for 15 mins at room temperature with 4% paraformaldehyde in 1 × PBS (pH 7.4) and then washed extensively with 1 × PBS. Cells were permeabilized in 1 × PBS containing 0.1% Triton X-100, washed twice in 1 × PBS containing 0.2% BSA (0.2% BSA–PBS). Cells were then stained for 45 min at room temperature with Alexa Fluor 488 phallotoxin (Molecular Probes, Inc., Eugene, OR) diluted 1:500 in 0.2% BSA–PBS. Cells were washed in 0.2% BSA–PBS and PBS as above, counter stained for 3 min at room temperature with DAPI (Santa Cruz Biotechnology, Inc., CA) and mounted for confocal microscopy (MRC-1000; Bio-Rad, CA).

2.5. Apoptosis assay

Apoptosis assays were performed as per manufacturer's instruction (BD-Pharmigen, NSW, Australia) and as previously described [14]. In brief, RAW264.7 cells were seeded (1×10^6 cells per well) into a 6-well plate containing 2 ml of complete medium. Cells were allowed to adhere overnight at 37 °C before exposure to varying doses of naringin for a further 24 h. Cells were harvested and resuspended in 0.5 ml of Annexin V Binding Buffer. Microfuge tubes containing resuspended cells (100 μl), Annexin V-PE (5 μl) and 7-Amino-Actinomycin (7-AAD) (5 μl) were gently vortexed and then incubated for 15 min at room temperature in the dark. Binding buffer (400 μl) was added to each tube and within 1 h, 10 000 cells were analyzed by flow cytometry (Becton Dickinson FACSCalibur®). Results were expressed as a percentage of apoptotic cells within the population.

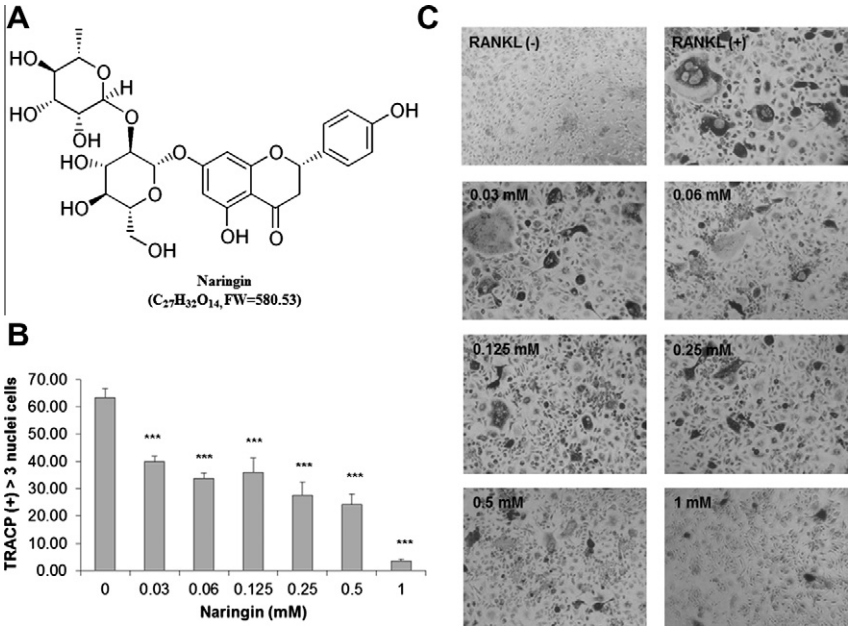

Fig. 1. Naringin inhibits RANKL-induced osteoclastogenesis in BMM cultures. (A) Chemical structure of naringin. (B) Freshly isolated BMMs were cultured in the presence of M-CSF and RANKL with different concentrations of naringin. Seven days post-culture, cells were fixed with 4% paraformadehyde followed by TRACP staining. Dose-dependent effect of naringin on the formation of TRACP(+) OCL cells (***P-value < 0.001). (C) Light microscope images depicting the dose-dependent effect of naringin on RANKL-induced osteoclast formation. A representative example of more than three experiments is shown.

2.6. Bone resorption pit assay

Human giant cell tumour (GCT) of bone samples was freshly isolated from patients who had undergone surgery at the Sir Charles Gairdner Hospital (Nedlands, WA, Australia). Tumour tissues were finely chopped in complete α-MEM and the resultant cell suspension was passed through a 100 μm nylon cell strainer (BD Bioscience, MA, USA). Approximately 200 OCL cells were seeded onto bovine bone slices in the presence and absence of naringin. After culturing for 24 h at 37 °C, bovine bone slices were incubated for 2 h in 2 M NaOH and OCL cells were removed by mechanical agitation and sonication. Resorption pits were visualized under Philips XL30 scanning electron microscope (S.E.M.) and the percentage of bone surface area resorbed quantified using Scion Image software (Scion Cooperation, National Institutes of Health) [12,13].

2.7. RNA isolation and Reverse transcription (RT)-PCR

Total RNA was isolated with a commercially available RNA extraction kit (Qiagen, Victoria, Australia). For RT-PCR, single stranded cDNA was prepared from 2 μg of total RNA isolated using reverse transcriptase with oligo-dT primer [15]. All PCR was carried out using 2 μl of each cDNA using cycling parameters 94 °C, 45 s; 58 °C, 45 s; and 72 °C, 45 s for 30 cycles (except for calcitonin receptor, TRACP and DC-STAMP whose annealing temperature was 60 °C) with primers designed against the following mouse sequences: Cathepsin K (forward: GGG AGA AAA ACC TGA AG; reverse: ATT CTG GGG ACT CAG AGA GC), calcitonin receptor: (forward: TGG TTG AGG TTG TGC CCA; reverse: CTC GTG GGT TTG CCT CAT C), TRACP: (forward: TGT GGC CAT CTT TAT GCT; reverse: GTC ATT TCT TTG GGG CTT), V-ATPase d2: (forward: ATG CTT GAG ACT GCA GAG; reverse: TTA TAA AAT TGG AAT GTA GCT), DC-STAMP: (forward: CTT GCA ACC TAA GGG CAA AG; reverse: TCA ACA GCT CTG TCG TGA CC) and acidic ribosomal phosphoprotein (36B4), which served as an internal control: (forward: TCA TTG TGG GAG CAG ACA; reverse: TCA ACA GCT CTG TCG TGA CC) [12]. Fifteen microliters of each PCR product were separated on a 1.5% agarose gel containing ethidium bromide and visualized and photographed under ultra-violet light.

2.8. NF-κB luciferase reporter gene assay

To examine NF-κB activation, RAW264.7 cells stably transfected with a luciferase reporter gene (p-NF-κB-TA-Luc) [16], were plated in 24-well plates at a density of 1×10^5 cells/well and pre-treated with naringin for 1 h, followed by GST-rRANKL (100 ng/ml) stimulation for 8 h. Cells were harvested and luciferase activity measured using the Promega Luciferase Assay System according to manufacturer's instructions (Promega).

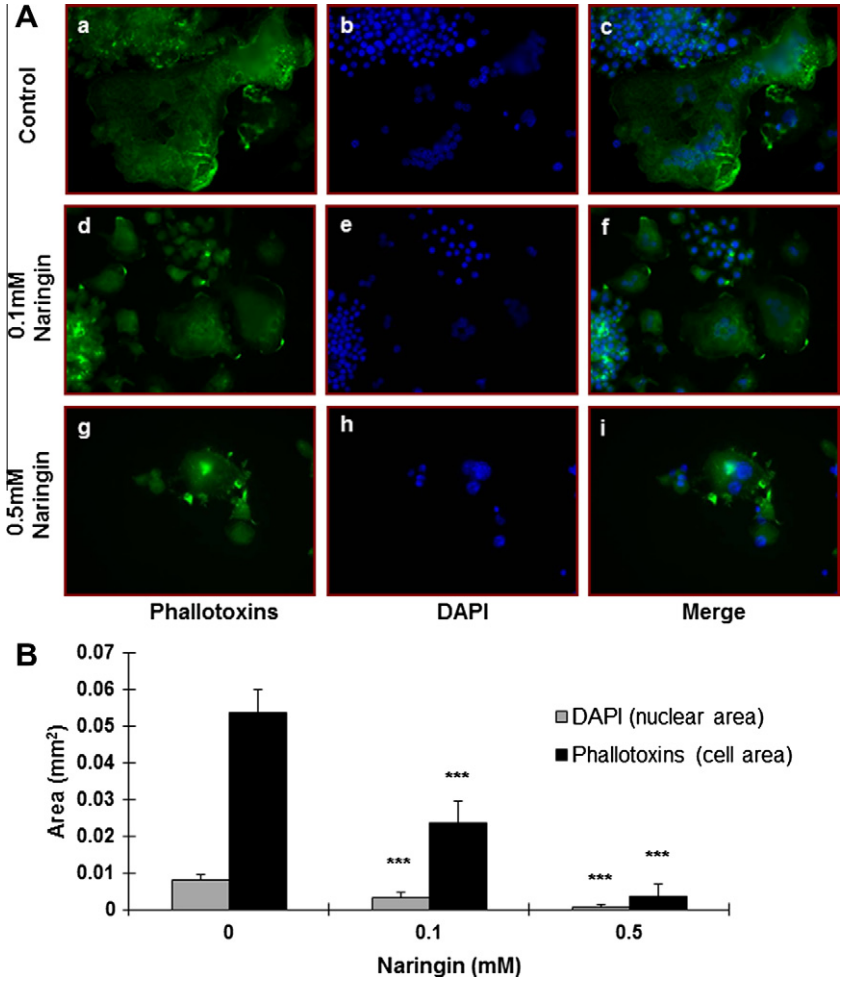

Fig. 2. Naringin decreases the size of OCL cells. RAW264.7 cells were cultured in the presence of RANKL with naringin in a six-well plate pre-seeded with glass coverslips. After 5 days of culture, cells were fixed with 4% paraformaldehyde and double stained with DAPI (nuclear staining) and phallotoxins (F-actin structure) antibodies. (A) Confocal images of OCL cells left untreated (a–c), treated with 0.1 mM naringin (d–f) and 0.5 mM naringin (g–i). (B) Total cell spread area and nuclear area of OCL cells from an average of five randomly selected fields (400×) were measured and graphed (***P-value < 0.001).

2.9. Western blot analysis of IκB-α and phosphorylated ERK

Proteins were separated by SDS–PAGE gel and electroblotted onto nitrocellulose membranes (BioRad). Membranes were blocked with 5% (w/v) skim milk powder (SMP) in TBST (10 mM Tris, pH 7.5, 150 mM NaCl, 0.1% {v/v} Tween 20) and then probed with primary antibodies to phosphorylated forms of ERK, IκB-α and α-tubulin (Santa Cruz Biotechnology Inc.) in 1% (w/v) SMP in TBST. After three washes with 1 × TBS, membranes were incubated with HRP-conjugated secondary antibodies diluted 1/5000 in 1% (w/v) SMP in TBST. The membranes were then developed using the ECL system (Amersham Pharmacia Biotech, Sydney, Australia).

2.10. Statistical analyses

Data presented were representative results from a set of three independent experiments or the mean ± S.E.M. of those experiments. Student's *t* test was used to analyze statistical significance between a tested group with its respective control group. A *P*-value of <0.05 was considered to be statistically significant.

3. Results

3.1. Naringin suppresses RANKL-induced osteoclastogenesis

To examine the effect of naringin on RANKL-induced osteoclastogenesis, we incubated RANKL-treated BMM with various concentrations of naringin and evaluated the formation of OCL cells. TRACP positive cells with more than 3 nuclei were scored as OCL cells. BMM cultured in the presence of M-CSF and RANKL form multinucleated TRACP-positive OCL cells (Fig. 1B). Addition of naringin into BMM cultures showed dose-dependent inhibition of osteoclast formation as measured by the TRACP positive multinucleated cells. Notably, OCL cells in cultures that were treated with naringin were smaller than control OCL cells (Fig. 1C). There was no observable cell loss (cell detachment) observed at concentrations tested up to 1 mM. To further evaluate the morphological changes, we examined the effect of naringin on the size of OCL cells during RANKL-induced osteoclast formation via the use of confocal microscopy. OCL cells in the presence or absence of naringin treatment were double-stained with phallotoxins and DAPI to allow for visualization of the cytoskeleton and nuclei respectively (Fig. 2A(a–c)). In comparison, naringin treated OCL cells were smaller in size and had fewer numbers of nuclei than the untreated control (Fig. 2A(d–i) and B). Naringin alone without the presence of RANKL did not stimulate osteoclast development (data not shown). These data thus suggest that naringin abrogates RANKL-induced osteoclastogenesis. To exclude the possibility that the observed inhibitory effect of naringin on osteoclastogenesis might be due to apoptosis, apoptosis assays and FACS analysis were carried out on osteoclast precursor cells stained with both Annexin V-PE and 7-AAD (Fig. 3A). Rates of apoptosis were low in RAW264.7 cells left treated or untreated with up to 1 mM of naringin. However, at concentrations of naringin greater than 5 mM, rates of apoptosis significantly increased, with more than 55% of cells undergoing apoptosis (Fig. 3B). These results suggest that the inhibition of osteoclastogenesis by naringin at doses up to 1 mM is not due to the induction of apoptosis.

3.2. Naringin attenuates osteoclastic bone resorption

To test the effect of naringin on osteoclastic bone resorption, equal number of Giant cell tumor of bone (GCT)-derived osteoclasts was seeded onto bovine bone slices and, after attachment naringin was added to the culture. Bone surfaces were retrieved after incubation for a further 24 h, processed for scanning electron microscopy, visualised and scored for bone resorption, as described in the methods. Treatment of cultures with naringin attenuated osteoclastic bone resorption (Fig. 4). Note that treatment of naringin resulted in very shallower resorption pits or reduced pit areas as compared to untreated control (Fig. 4A and B). Noteworthy, this impairment of bone resorption did not reflect cell death of OCL

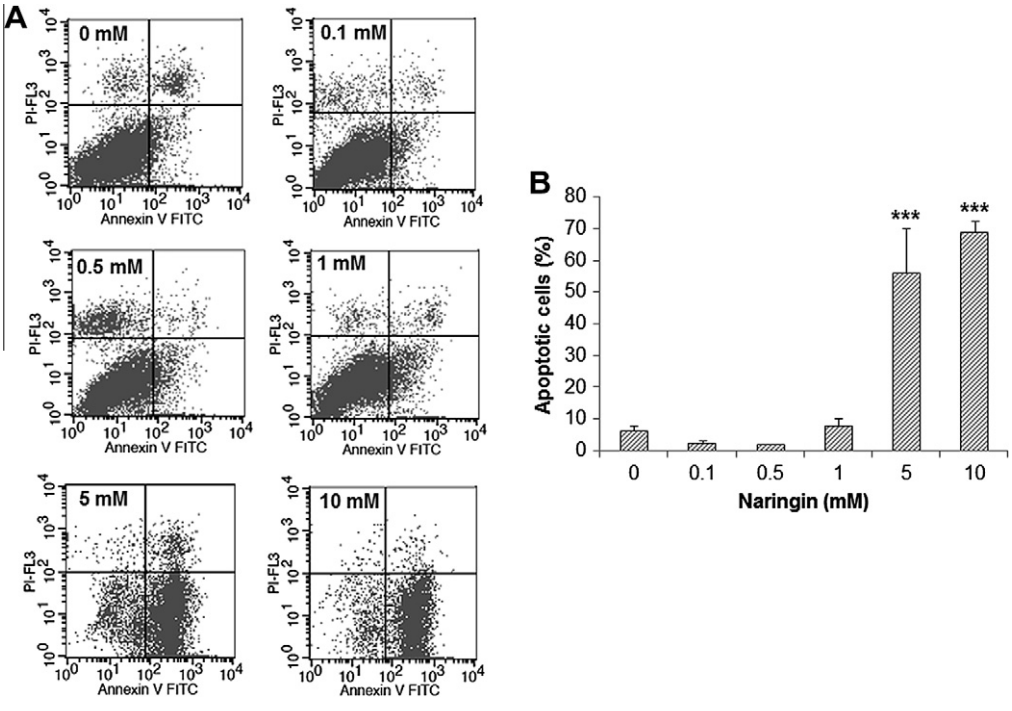

Fig. 3. Naringin dose dependently induces apoptosis of RAW264.7 cells. (A) RAW264.7 cells treated with varying doses of naringin for 24hrs were harvested, double-stained with Annexin V-PE and 7-AAD and 10 000 cells were subjected to analysis by flow cytometry. (B) Results, representative of three independent experiments, show the percentage of the total cells population displaying apoptosis (***P-value < 0.001, compared to the untreated controls).

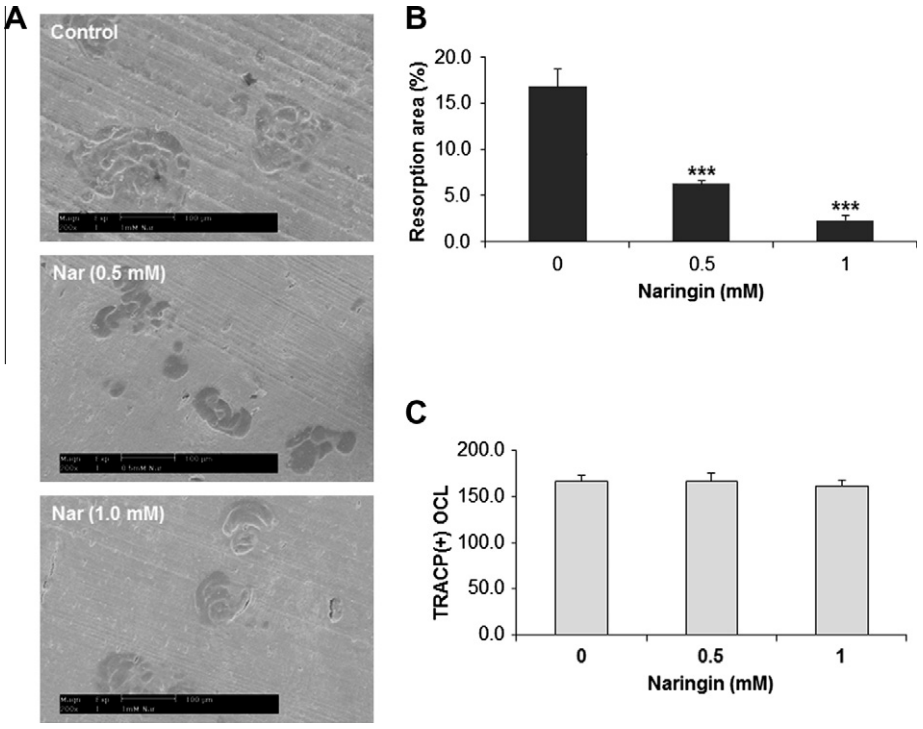

Fig. 4. Naringin abrogates bone resorption and induced apoptosis. Equal number of OCL cells (derived from patients presenting with GCT of bone) was seeded onto bovine bone slices and allowed to adhere to the surface before the addition of naringin at varying doses (0, 0.5 and 1 mM) for 24 h. (A) Representative S.E.M. images of bone resorption pits are shown. (B) The total areas of resorption pits were measured under S.E.M. and are presented graphically (***P-value < 0.001). (C) Total number of TRACP positive OCL cells per bone slice.

cells as no significant differences in the total number of TRACP-positive cells per bone slice were observed after the treatment of naringin (Fig. 4C). Taken together, these experiments conclude that naringin inhibits osteoclastic bone resorption.

3.3. Naringin suppresses RANKL-induced gene expression

To further elucidate the role of naringin on osteoclast differentiation, we examined its effect on the gene expression of cathepsin k, calcitonin receptor, TRACP, dendritic cell-specific transmembrane protein (DC-STAMP), V-ATPase d2 (d2), all marker genes of osteoclasts. BMM cultures were treated with M-CSF (25 ng/ml) and GST-rRANKL (100 ng/ml) in the absence or presence of naringin for 5 days followed by with total RNA isolation. Semi-quantitative RT-PCR was performed using primers for cathepsin k, calcitonin receptor, TRACP, DC-STAMP, d2 and 36B4. Naringin reduced the gene expression of osteoclastic gene markers cathepsin k, calcitonin receptor and TRACP in a dose dependent manner during osteoclastogenesis (Fig. 5A and B), consistent with its inhibitory effects on osteoclastogenesis and bone resorption. To add to this, naringin also suppressed osteoclast fusion gene (DC-STAMP and d2) expression.

3.4. Naringin suppresses RANKL-induced activation of NF-κB by preventing IκB-α degradation

To measure the inhibitory effect of naringin on NF-κB transcriptional activity, RAW264.7 cells that had been stably transfected with an NF-κB luciferase reporter construct [16] were stimulated with RANKL in the presence and absence of naringin. Twenty-four hours of RANKL (100 ng/ml) treatment alone, an increase in luciferase activity was observed. Pre-treatment (1 h) of cells with naringin prior to RANKL stimulation (8 h) resulted in a significant dose-dependent reduction in NF-κB luciferase activity (Fig. 6A). Surprisingly, naringin alone increased the NF-κB luciferase activity in a dose dependent manner, indicating a role for naringin in biphasic modulation of NF-κB signaling pathways.

To add to this, we investigated the effect of naringin on RANKL-induced. IκB-α degradation was observed at 10 and 20 min post-RANKL-stimulation with maximum degradation observed at 20 min as compared to unstimulated controls. The addition of naringin resulted in a suppression of IκB-α degradation, as seen at a concentration (0.5 and 1 mM) as well as time (10 and 20 min) dependent manner (Fig 6B). Interestingly, in the absence of RANKL, the basal levels of IκB-α were increased by naringin at 1 h and 20 min post-treatment (0.5 and 1.0 mM).

3.5. Naringin inhibits RANKL-induced ERK phosphorylation

To further explore pathways by which naringin regulates osteoclast differentiation and function, the effect of naringin on RANKL-induced ERK phosphorylation was examined in RAW264.7 cells that had been exposed to naringin. Western blot analyses demonstrated ERK phosphorylation after 15 mins of RANKL treatment, and naringin exhibited an inhibitory effect on RANKL-induced ERK phosphorylation (Fig. 7). In comparison, in the presence of naringin, the basal level of ERK1/2 was increased in the absence of RANKL stimulation.

4. Discussion

Animal studies have found that naringin can protect against retinoic acid-induced osteoporosis in Sprague Dawley rats [9] and improve bone quality in orchidectomized male rats [10]. Interestingly, in this study, we showed that naringin inhibits osteoclast formation and bone resorption via the suppression of RANKL-induced activation of NF-κB and ERK. These data provide the mechanistic explanation, at least in part for the protective effect

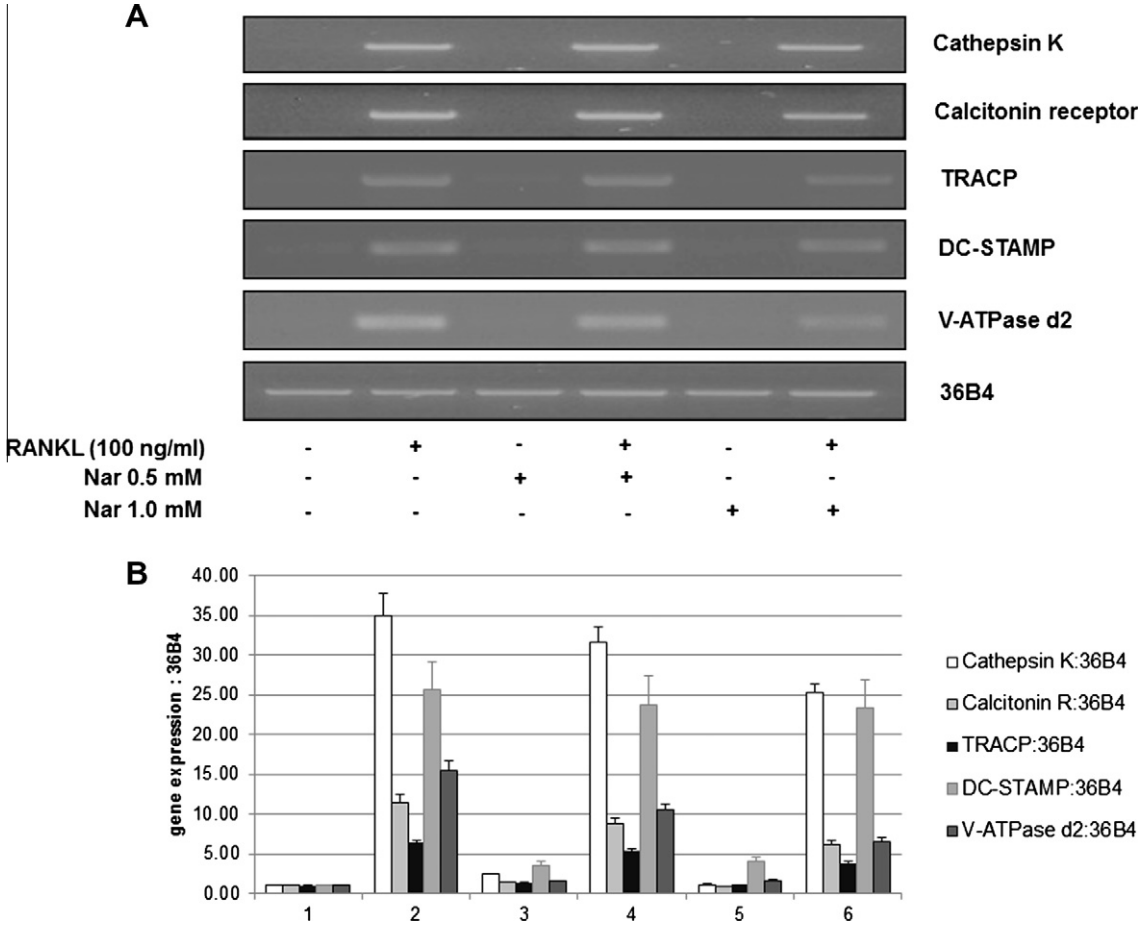

Fig. 5. Naringin dose-dependently reduces RANKL-induced gene expression (A, B). BMM cells were seeded onto six-well plates in the presence and absence of RANKL (100 ng/ml) for 5 days with various doses of naringin (0, 0.5 and 1 mM). Total RNA was isolated and cDNA was synthesized using 2 μg of total RNA with oligo-dT. PCR amplification was performed using specific primers for cathepsin K, calcitonin receptor, TRACP, DC-STAMP, d2 and 36B4 (house-keeping) genes. (A) PCR products were separated on 1.5% agarose gels. (B) The relative levels of gene expression are shown as the ratios of cathepsin k, calcitonin receptor, TRACP, DC-STAMP, d2 to 36B4 genes.

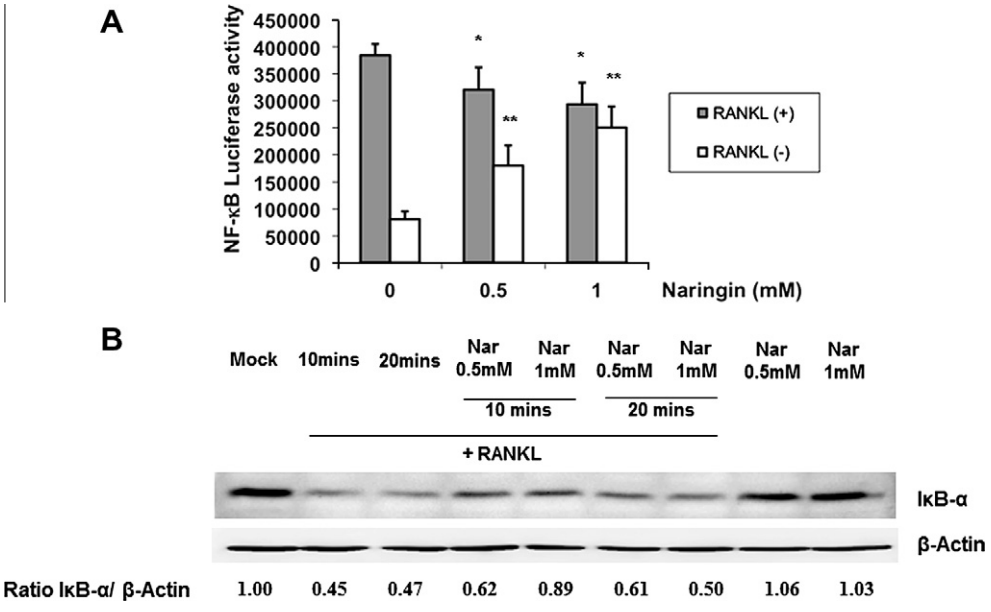

Fig. 6. Naringin suppresses RANKL-induced NF-κB activation, prevents IκB-α degradation. (A) RAW264.7 cells stably transfected with the 3kB-Luc-SV40 reporter gene were pre-treated with varying doses of naringin for 1 h followed by RANKL (100 ng/ml) stimulation. Luciferase activity in the lysates was determined after 8 h of RANKL stimulation. Each bar represents the mean ± SE from triplicate wells (*P-value < 0.05, **P-value < 0.01). (B) RAW264.7 cells were pre-treated with naringin for 1 h prior to RANKL (100 ng/ml) stimulation for 0, 10 and 20 min. Proteins extracted from whole cell were separated and transferred onto nitrocellulose membranes, which were then blocked and probed sequentially with antibodies to IκB-α and β-actin. Bands were visualized by ECL and semi-quantified by densitometry. Results shown are a representation of one of three experiments performed. The levels of IκB-α proteins are shown as a ratio to β-actin.

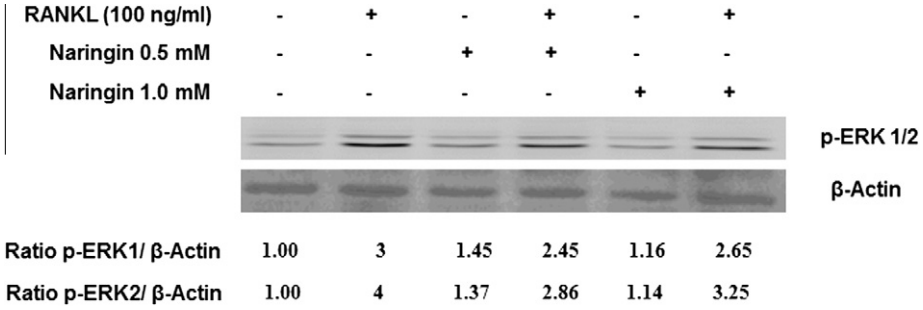

Fig. 7. Naringin inhibits RANKL-induced ERK phosphorylation. RAW264.7 cells were pre-treated with naringin for 1 h prior to RANKL (100 ng/ml) stimulation for 0 and 15 mins. Proteins extracted from whole cell were separated and transferred onto nitrocellulose membranes, which were then blocked and probed sequentially with antibodies to phosphorylated ERK and β-actin. Bands were visualized by ECL and semi-quantified by densitometry. Results shown are a representation of one of three experiments performed. The levels of phosphorylated ERK proteins are shown as a ratio to β-actin.

of naringin against bone loss. Understanding the cellular and molecular mechanism by which this natural compound inhibits osteoclasts might provide invaluable information for the treatment of osteolysis.

During RANKL-induced osteoclastogenesis, RANKL binds with RANK resulting in the recruitment of TRAF6 and the activation of NF-κB and MEK kinases [17–19]. NF-κB signaling has been shown to play an important role in osteoclastogenesis [3]. NF-kappaB p50−/− and p52−/− double knockout mice exhibit severe osteopetrosis due to failure of osteoclast formation [20,21]. Therefore suppression of NF-κB activation would play an important role in osteoclast formation. Interestingly, in the present study, naringin was shown to reduce RANKL-induced NF-κB-activity, simultaneously with inhibition of RANKL-induced differentiation of osteoclasts. Intriguingly, naringin appeared to increase the basal level of NF-κB activity in the absence of RANKL stimulation (Fig. 6A). However, naringin alone also appeared to increase the IκB-α levels, 1 h and 20 min post-treatment in a time-dependent manner (Fig. 6B). This could represent the resynthesis of IκB-α following the activation of NF-κB accompanied with the initial IκB-α degradation. The MEK/ERK also regulates the differentiation of RAW264.7 cells into osteoclast-like cells [22–24]. We have demonstrated that naringin prevents RANKL-induced phosphorylation of ERK, outcomes which might also affect osteoclast differentiation and survival. The effect of naringin on MEK/ERK has not been documented in other cell types, representing novel data that requires further investigation. Interestingly, in line with our findings from this study, naringin has been shown to inhibit TNF-induced reactive oxygen intermediate (ROI) generation and NF-κB, indicating that naringin might serve as a potent drug for anti-inflammatory and antioxidant therapy [25]. These multiple effects of naringin could further explain the underlining mechanism of action of naringin in osteoclastogenesis and bone resorption.

Previous studies have shown that naringin suppresses TNF production induced by LPS [26,27]. Interestingly, naringin, at a similar concentration used in this study, also suppressed LPS induced production of nitric oxide (NO) and the expression of inflammatory gene products, including inducible NO synthase (iNOS), TNF-alpha, inducible cyclooxygenase (COX-2) and interleukin-6 (IL-6) via the inhibition of NF-κB [28]. Since iNOS, COX-2, TNF-α and IL-6 are positive regulators for osteoclastogenesis [29,30], and the expression of a battery of these genes is induced by NF-κB [31], the inhibition of NF-κB by naringin could result in the suppression of the expression of these genes and might contribute to its inhibitory effect on osteoclast formation and bone resorption. Understanding the pharmacological mechanisms of naringin in bone biology will be important in developing an effective approach to prevent and treat osteolysis.

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